



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> SOLID PHASE SYNTHESIS  <b>(57) Abstract</b>  The present invention relates to a support system for solid phase synthesis of oligomers, such as oligonucleotides, wherein the starting compound is bound to the support via a disiloxyl linkage. Furthermore, the invention relates to a method for synthesis of oligonucleotides on a solid support. The support system comprises a stable disiloxyl linkage providing high nucleoside loadings to the support and the method allows convenient non-laborious oligomer synthesis.		

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## SOLID PHASE SYNTHESIS

### Technical field

The present invention relates to a support system for solid phase synthesis of oligomers, such as oligonucleotides. Furthermore, the invention relates to a method for synthesis of oligonucleotides on a solid support.

### Background of the invention

Oligonucleotides are polymers built up by polycondensation of ribonucleoside (RNA) or deoxyribonucleoside (DNA) phosphates.

Oligonucleotides can be assembled by repetitive addition of nucleotide monomers using solid-phase methods. Since the introduction of solid-phase synthesis [R.B. Merrifield, J. Am. Chem. Soc. 85 (1963) 2149], the following requirements have been worked out: (1) The solid support must be insoluble and preferably unswellable in the solvent used. (2) Functional groups on the solid support must allow covalent binding of the first nucleoside in a reproducible manner. (3) The solid support must be chemically inert to all reagents used during synthesis and deprotection. The most commonly used supports are controlled pore glass beads (CPG), silica, or polystyrene beads.

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Below the synthesis cycle of the commonly used phosphoramidite method is described:

1. Deprotection of the 5'-hydroxyl group in order to generate the parent hydroxyl compounds. This is normally done by treatment of the support with di- or trichloroacetic acid in an organic solvent (for removal of protecting groups).
2. The support is washed in order to remove traces of acid.
- 35 3. The 5'-hydroxyl group is reacted with the 3'-phosphoramidite moiety of a properly protected incoming

nucleotide (A, C, G or T) in the presence of an activator (e.g. tetrazole) to form a 3'-5'-phosphite triester.

4. Excess reagents are removed by washing with an appropriate solvent.

5 5. Unreacted 5'-hydroxyl groups are blocked as acetates (capping).

6. The capping reagent is removed by washing.

7. The phosphite triester is then oxidated to the corresponding phosphate triester. This is normally done by  
10 the action of aqueous iodine.

8. The oxidation reagents are removed by washing.

The process is repeated until the desired oligonucleotide sequence has been synthesized. After synthesis, all  
15 protecting groups are removed and the oligonucleotide is cleaved from the solid support.

In the synthesis, defective oligonucleotides are produced as a consequence of several effects, prominently premature  
20 termination of synthesis, followed by capping, which results in 5' truncated molecules, and depurination during the synthetic cycles that is followed by strand scission during deprotection. Recently, attention has also been directed at the appearance of shorter, internally deleted products - so  
25 called n-1 and n-2 fragments [Temsamani et al, (1995), Nucleic Acids Research 23(11), 1841-1844]; [Fearon et al, (1995) Nucleic acids Res., 23(14), 2754-2761].

The need for pure oligonucleotides is exemplified by the  
30 requirement for high quality products in antisense therapy [Gelfi et al, (1996), Antisense and Nucleic Acid Drug Development, 6, 47-53], in routine diagnostics applications, or for physicochemical and structural studies [Agback et al, (1994) Nucleic Acids Res, 22(8), 1404-12]. Also in molecular  
35 cloning impure oligonucleotides frequently reduce efficiency and complicate interpretation of results [McClain et al,

(1986) Nucleic Acids Res. 14(16), 6770]; [Nassal, (1988) Gene, 66(2), 279-94].

Preparative gel electrophoresis provides the best resolution for purification of oligonucleotides. The method is however laborious, often leading to considerable loss of material, and it is poorly suited for automation and scale-up.

Chromatographic separation can solve some of these problems, offering a potential for scale-up with minimal losses and using fully automatized instruments. These positive aspects are off-set by the rather poor resolving power of most chromatographic systems. As a partial solution to this problem chromatographic separation of oligonucleotides labeled with affinity tags has been used. The commonly used trityl-on oligonucleotide separation on reversed-phase columns, or capture of 5'-thiol labelled or biotinylated oligonucleotides on respective thiol-affinity [Bannwarth et al, (1990), Helv. Chim. Acta, 73, 1139-1147] or avidin columns [Olejnik et al, (1996), 24(2), 361-366] offer the possibility to isolate fragments with intact 5'-ends. However, the 5' part of depurinated molecules notoriously contaminate oligonucleotides purified by this method.

A mild basic system has been proposed for partial deprotection and cleavage of apurinic-sites with the oligonucleotides still bound to the solid support. In this manner the 5' ends of depurinated molecules can be discarded before the oligonucleotides are released from the support, followed by isolation of molecules with intact 5' ends [Horn et al, (1988), Nucleic Acids Res, 16(24), 11559-71]. In practice, this strategy was accompanied by a substantial loss of products, due to inadvertent release of oligonucleotides during cleavage of depurinated sites.

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In WO92/09615 there is described the use of an alkoxysilyl group as a linker of the oligonucleotide to the support.

This linker is inert during the synthetic cycles and it resists conditions that cleave apurinic sites. The linker is finally cleaved from the solid support with tetra butyl ammonium fluoride (TBAF) to obtain, after reversed-phase separation of DMTr-containing material, an oligonucleotide with both 3'- and 5'- ends intact. However, synthesis of this support was laborious and inconvenient. Due to low reactivity of the functional group of the linker the degree of substitution of the support becomes low which leads to insufficient nucleoside loadings of the support. Thus, this method is not suitable for preparation of support useful for large scale synthesis.

#### Summary of the invention

According to a first aspect, the invention provides a support system for solid phase synthesis of oligomers. The support system comprises a support, a linker and a starting compound of the oligomer. The starting compound is bound to the support via a disiloxyl linkage. The disiloxyl function is linked to a hydroxyl group on the support. The functional groups connected to the disiloxyl group are very reactive allowing for reproducible and controlled loading of the starting compounds.

The support system of the invention is easier to produce compared to prior art systems and provides for high loadings to the support. According to the invention high loading values are obtained for the starting nucleoside. These loadings, often higher than 200  $\mu\text{mol/g}$ , are required for cost-effective large scale synthesis.

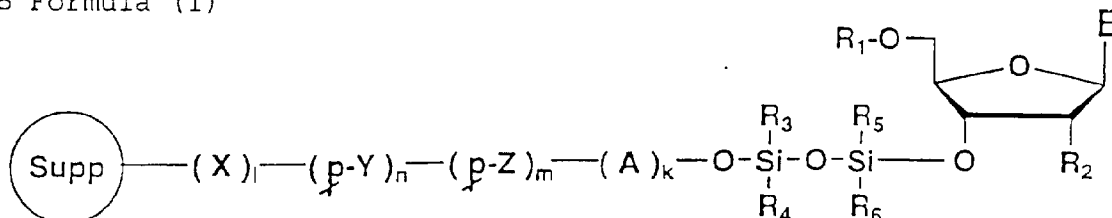
The linkage is inert during the synthesis cycles and resists conditions that cleave apurinic sites.

In a preferred embodiment, the starting compound is a nucleoside and the solid phase synthesis is used for the synthesis of oligonucleotides.

Supports with immobilized oligonucleotides can be used as hybridization affinity matrices. Some possible applications of such supports are: purification of DNA-binding proteins, 5 affinity purification of plasmids, as a support for gene assembly (from oligonucleotides) and for diagnostic purposes, etc.

In the new support system of the present invention the first 10 nucleoside is bound to the support via a disiloxyl linkage and the system is preferably represented by the following formula (I).

15 Formula (I)



wherein

B is a ribonucleoside or deoxyribonucleoside base;  $R_2$  is -H, -OH, or  $OR_7$  in which  $R_7$  is a protecting group;  $R_1$  is a 25 protecting group;  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$  taken separately each represent alkyl, aryl, cycloalkyl, alkenyl, aralkyl, cycloalkylalkyl, alkyloxy, aryloxy, cycloalkyloxy, alkenyloxy and aralkyloxy; Supp is a solid support; X is an anchoring group used for covalent bonding to the support;

30  $(p-Y)_n$  and  $(p-Z)_m$  are oligophosphotriester linkers, wherein p represents a phosphotriester, Y and Z are independently selected from a nucleoside and a rest of a diol, A is an aliphatic or aromatic group, n is a number between 0-50, preferably 0-10, and k, l, m are each a number of 0 or 1,

35 with the proviso that when m and n are 0 then l and k are 0 and with the proviso that when m = 1 then k is 0 and X is O or S.

The protecting groups  $R_1$  and  $R_7$  are protecting groups usually used for protection of 5' and 2' position of ribo- and deoxiribonucleosides.  $R_1$  may be selected from a trityl, 5 monomethoxy trityl, dimethoxytrityl, pixyl or other higher alkoxy-substituted trityl- protecting groups.

$R_7$  may be selected from tertbutyldimethylsilyl (TBDMS), methoxytetrahydropyranyloxy (MTHP), tetrahydropyranyloxy, methyl or allyl.

10

In a preferred embodiment of the invention  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$  are isopropyl. The choice of  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$  is dictated by stability vs. lability requirements of the disiloxyl linker. It is known that these properties can easily be controlled 15 by modifying electron donating parameters of the substituents.

X can be any anchoring group, preferably O, S or an amide function, provided it is stable to the conditions used under 20 synthesis and the reagent to cleave apurinic sites. The same proviso applies also to the  $(p-Y)_n$  linker and  $(p-Z)_m$ . Thus, there are no other restrictions on Y and Z.

Z is exemplified by a tetraethylene glycol residue.

25

A wide range of porous as well as non-porous solid supports can be used as supports in methods according to the present invention. The group of preferred supports includes cross linked polystyrenes, silica, polysaccharides, crosslinked 30 polysaccharides and various glasses.

When the oligophosphotriester linker  $(p-Y)_n$  is present in the above formula a support is provided resulting in even greater oligonucleotide purity than without said linker. By 35 preparing the supports through a series of synthetic cycles before addition of the cleavable disiloxyl linker and synthesis of the desired oligonucleotide, any nonspecific



sites of synthesis will be neutralized. This linker gives improved contact between the starting nucleoside and an incoming reagent and ensures that also oligonucleotides starting from sites not intended for synthesis on the support none the less contribute to the production of the desired oligonucleotide.

According to a second aspect the invention provides a method for oligonucleotide synthesis on a solid support Supp. The method comprises the steps:

- (i) preparing a support system as defined above;
- (ii) condensation of nucleotides onto the first nucleoside of the support system to synthesize an oligonucleotide;
- (iii) removal of all protecting groups on the oligonucleotide except the 5'-protecting group, and cleavage of apurinic sites formed during acid-catalysed deprotection;
- (iv) cleavage of the full length product from the support; and
- (v) purification of the oligonucleotide.

In step (i) an oligophosphotriester linker  $(p-Y)_n$  is synthesized on the solid support and a starting nucleoside is bound to the linker via a  $(p-Z)_m$  linker and a disiloxyl group.

According to one embodiment of the method, the disiloxyl linkage is cleaved selectively according to known methods (Markiewicz W.T., 1979, Journal of Chemical Reserche (M) 0181-0197) with a compound containing fluoride ions, before step (v). A preferred fluoride containing compound is tetra alkyl ammonium fluoride. Trityl ammonium hydrogen fluoride is also suitable. The purification is performed by reversed phase chromatography, using the 5'-protecting group as an affinity handle.

According to an alternative embodiment of the method according to the invention, step (v) is performed by

exonuclease treatment whereby non-protected oligonucleotides will be digested. This embodiment is especially suitable for in situ synthesis where chromatography is not possible.

## 5 Detailed description of the invention

The invention will be described more closely below in association with an Experimental part and the accompanying drawings, in which

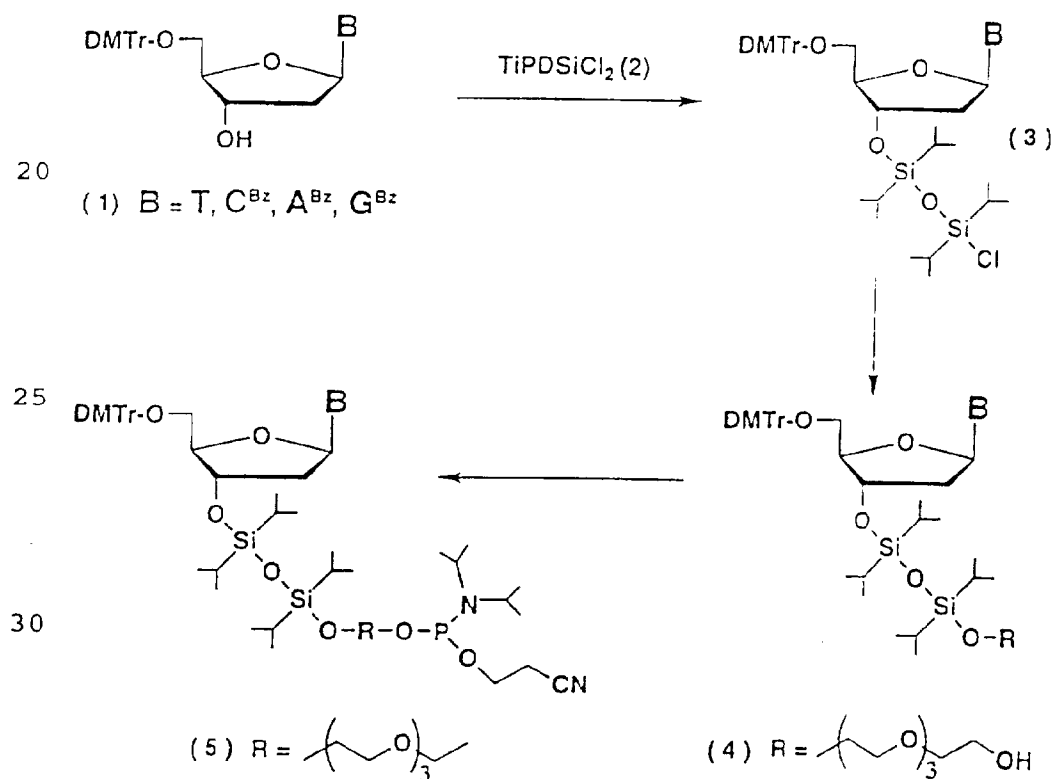
- 10 **Figure 1** shows starting points for oligonucleotide synthesis with or without an oligonucleotide linker (14 and 13, respectively) and the support after accomplished synthesis (15).
- 15 **Figure 2** shows the results of synthesis of a purine rich (GA)<sub>40</sub> oligonucleotide. **A)** A reverse phase HPLC chromatogram showing the trityl containing material. The product from a standard support appears as a much broader peak with a large proportion of material appearing later
- 20 compared to the sharp and symmetrical peak from the novel support. **B)** Products collected from the peaks in **A** were 5' labelled and separated on a denaturing polyacrylamide gel. An autoradiogram showing the oligonucleotide obtained using a standard support on the top and material
- 25 synthesized on the novel support below. **C** and **D)** The same gel as in **B** was scanned on a Phosphorimager (Molecular Dynamics). The data are presented as line graphs with standard support in **C** and the novel support in **D**. The n-1 and n-2 peaks are marked in **C**.
- 30 **Figure 3** shows circularization of a 91-mer padlock probe. The term "padlock probe" means a probe which is able to circularize on its target sequence and is described in Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdahry, B.P. and Landegren, U. (1994) Science, 265 (5181), 2085-8, to which reference is made. The oligonucleotide was labelled at the 5'-end and the two

ends were ligated using an excess of a complementary oligonucleotide as template. The reaction was performed for several cycles of denaturation and ligation using a thermostable ligase. Lane 1: no ligase. Lanes 2,3 and 4: 1, 2 and 3 cycles of ligation, respectively. All full length (n) oligonucleotides were circularized in the first round of ligation.

The preparation of the support system according to the invention can be performed according to scheme 1 illustrated below:

SCHEME 1

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This procedure comprises the following steps:

1. Silylation of the 5'-dimethoxytritylated and appropriately protected nucleosides using 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane in dry pyridine with  
5 addition of imidazol.
2. Reacting of the formed monochloroderivative (3) with an diol e.g. tetraethylene glycol to obtain derivative (4).
3. Introduction of phosphoramidite function to the free hydroxyl group located at the end of the 3'-linker.

10

According to the invention the homobifunctional, readily available reagent 1,3-dichloro-1,1,3,3-tetraisopropyl disiloxane (2) (scheme 1) was used. In other words, the reagent used in the present invention has two reactive  
15 functions which enables high and controllable substitution.

A disiloxyl residue was applied as a stable linker function between a nucleoside and a solid support. The  
20 reactive intermediates (3) or (5) were synthesized in one or three steps, respectively according to Scheme 1 above.

No attempts were made to isolate intermediate (3), therefore silylation of appropriately protected  
25 nucleosides (1) were performed using only a slight excess of (2). The addition of an excess of an 13-atoms long chain diol (tetraethyleneglycol) resulted in the formation of an unsymmetrical disiloxyl derivative (4), used subsequently for the synthesis of phosphoroamidite (5).

30

The invention requires a support functionalized with hydroxyl groups linked to the support through nonhydrolyzable bonds. Mono R, hydroxyalkyl-derivatized polystyrene particles (from Pharmacia) was used without  
35 modifications. Derivatization of CPG proceeded according to Scheme 2, below.

## SCHEME 2

5

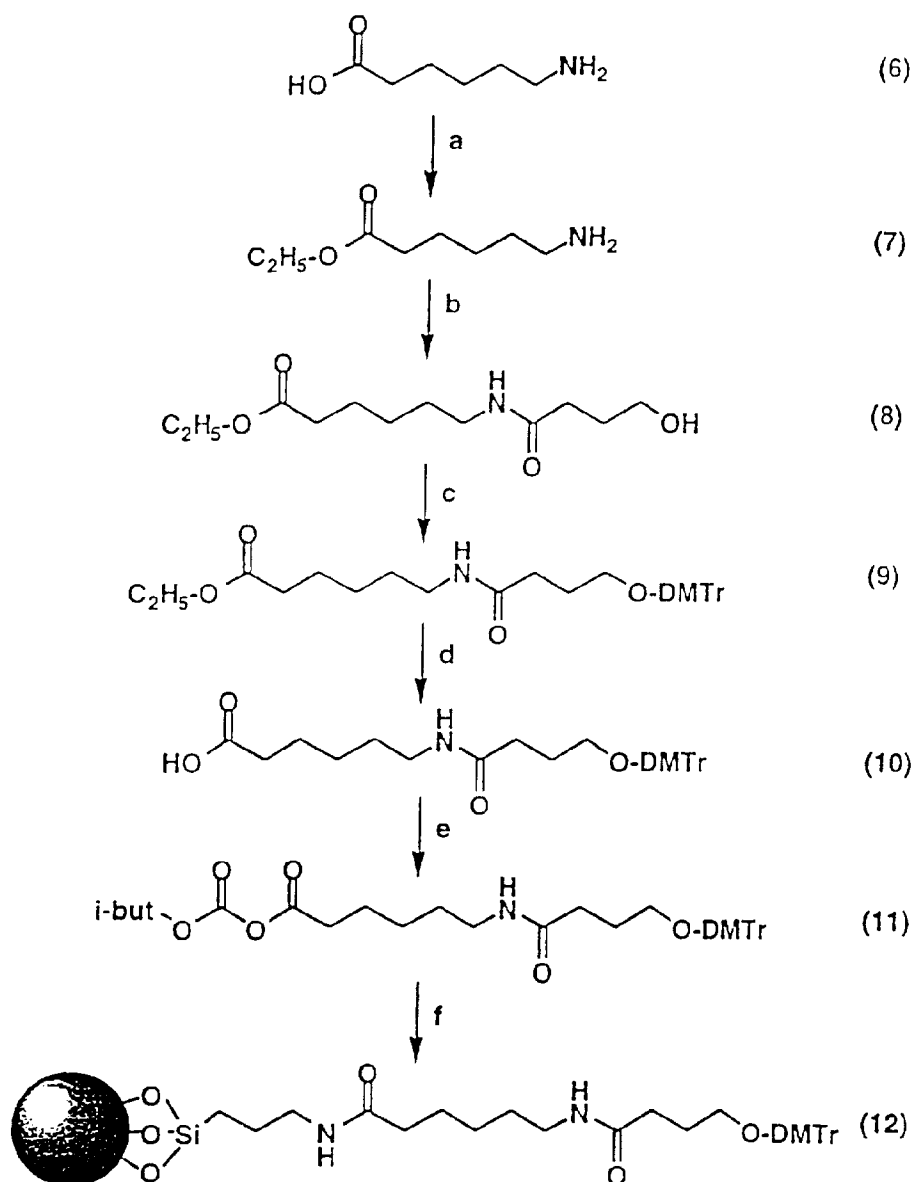
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a) thionyl chloride, ethanol; b)  $\gamma$ -butyrolacton, triethylamine;  
 c) dimethoxytrityl chloride, pyridine; d) NaOH, triethylamine,  
 e) isobutyl chloroformate; f) aminopropyl-CPG

This scheme includes the following reactions:

- a) refluxing of 6-aminohexanoic acid in dry ethanol with addition of thionyl chloride to achieve esterification of carboxyl group.
- 5 b) acylation of the amino group with  $\gamma$ -butyrolactone to form compound (8).
- c) protection of the introduced hydroxyl group with dimethoxytrityl group performed by DMTrCl in dry pyridine.
- d) hydrolysis of the ester group with NaOH and formation of  
10 the triethylammonium salt of the resulting acid.
- e) activation of the carboxyl group by preparing a mixed anhydride upon addition of isobutyl chloroformate.
- f) coupling of the activated reagent to the CPG support derivatized with amino functions to form support (12).

15

The CPG was activated by conventional aminopropylsilanizing [Pon, (1993) In Agraval, S. (ed.), Methods in Molecular Biology. Protocols for Oligonucleotides and Analogs. Humana Press Inc.]. Prior to  
20 further derivatization, the amino CPG support was treated with trichloroacetic acid in dichloromethane according to Pon [Pon, (1993), supra]. Compound (10) was prepared in high yield starting from an inexpensive 6-aminohexanoic acid and  $\gamma$ -butyrolactone. It was conveniently converted to  
25 the mixed anhydride (11) in a reaction with isobutylchloroformate, and immediately used for coupling to CPG, see Scheme 2. Using different ratios of (11) and a solid support, loadings ranging from 15 to 60  $\mu\text{mol/g}$  were obtained. The derivatized support was exhaustively capped  
30 with acetic anhydride and silanized with trimethylsilylchloride, TMSCl.

#### EXPERIMENTAL PROCEDURES

Controlled-pore glass (CPG) (1000 Å) was obtained from CPG  
35 Inc. (Fairfield, USA) and was aminopropylsilanized according to Pon et al. [Pon, 1993, supra]. Crosslinked polystyrene

particles (10 µm diameter) derivatized with hydroxylalkyl functions (Mono R) (from Pharmacia, Uppsala, Sweden).

Oligonucleotide syntheses were performed either on an ABI 5394 DNA Synthesizer (Perkin Elmer) or on a Gene Assembler Plus (Pharmacia Biotech AB) instrument. Analytical liquid chromatography of the synthesized oligonucleotides was performed on a Hitachi-Merck La Chrom HPLC system equipped with a LiChrospher RP 18 (5 mm) column (Merck) and using a linear gradient of solvent A: acetonitrile 5 % v/v in triethylammonium acetate 0.1 M, pH 7.0 and solvent B: acetonitrile 40 % v/v in triethylammonium acetate 0.1 M, pH 7.0. Preparative separations were made on an FPLC system (Pharmacia Biotech AB) using a reversed-phase Pep RPC 10/10 column (Pharmacia) and the above solvent gradient.

#### Example 1: Synthesis of nucleoside derivatives

##### 5'-Dimethoxytrityl-3'-O-1,1,3,3-tetraisopropyl-3-

20 tetraethylen-glycoloxy-disiloxyl thymidine (4) (B=T).

5'-DMTr-thymidine (1) (1.30 g, 2.3 mmol) and imidazol (0.32 g, 4.8 mmol) were dried by coevaporation with dry pyridine and dissolved in 20 ml of dry pyridine. 1,3-Dichloro-1,1,3,3-tetraisopropyl disiloxane (2) (0.75 g, 2.4 mmol) was 25 added and the mixture was stirred at 20°C for 3 hrs to achieve complete consumption of starting material. Tetraethylene glycol (3.9 g, 23 mmol) was added to the formed compound (3) (see Scheme 1) and the mixture was stirred for 6 hr. Pure compound (4) was isolated as an oil 30 (1.62 g, 72%), following standard bicarbonate work-up, extraction with dichloromethane, evaporation of the organic phase, and flash column chromatography. <sup>1</sup>H-NMR (CDCl<sub>3</sub>): 0.85-1.05 (m, 28 H), 1.43 (s, 3 H), 2.25-2.42 (m, 2 H), 2.85 (s, broad, 1 H), 3.27-3.52 (dd, 2 H), 3.54-3.76 (m, 12 H), 35 3.79 (s, 3 H), 3.80-3.85 (m, 2 H), 4.09 (m, 1 H), 4.68 (m, 1

H), 6.40 (t, 1 H), 6.83 (d, 4 H), 7.23-7.40 (m, 9 H), 7.63 (d, 1 H), 9.19 (s, broad, 1 H).

The other three nucleoside derivatives were obtained in a similar manner.

#### Example 2: Synthesis of phosphoramidites

##### Synthesis of a (2-cyanoethyl) N,N-diisopropylphosphoramidite

10 (5) (B=T). The thymidine derivative (4) (1.50 g, 1.53 mmol) was dried by coevaporation with toluene (20 ml) and dissolved in anhydrous dichloromethane (15 ml). To this magnetically stirred solution dry triethylamine (0.85 ml, 6.0 mmol) was added followed by 2-cyanoethyl-N,N-diisopropylaminophosphochloridate (710 mg, 3.0 mmol). After 15 min stirring at 20°C, TLC showed consumption of all starting material and formation of a single product. The reaction mixture was quickly partitioned between saturated aqueous sodium bicarbonate and dichloromethane and extracted 20 with dichloromethane (2x50 ml). The residue obtained after evaporation of the organic phase was dried by coevaporation with toluene and purified on a short silica gel column, prepared and eluted with CH<sub>2</sub>Cl<sub>2</sub>/Et<sub>3</sub>N 9/1 v/v. Fractions containing the desired product were combined, evaporated in 25 vacuo, coevaporated with dry

triethylamine, and dried in high vacuum to yield 1.57 g (87 %) of an oil; <sup>31</sup>P-NMR (CDCl<sub>3</sub> + 2 drops of triethylamine) 148.61 ppm.

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The remaining amidites were prepared as above.

#### Example 3: Construction of hydroxyalkyl-derivatized CPG support (14) (See Fig. 1)

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Asymmetrical anhydride (11) (see Scheme 2) was used for derivatization of aminopropylated CPG. The substitution



level was analyzed based on DMTr cation release as described in [Gait, (ed) (1984) Oligonucleotide synthesis; a practical approach.. IRL Press]. CPG derivatized to the extent of 28  $\mu\text{mol/g}$  was selected for further experiments. Several DNA synthesis columns were loaded with this support (ca 10 mg each), and they were subjected to 10 coupling cycles with a standard thymidine amidite, followed by coupling of one of the nucleoside amidites (5) to obtain support (14). These supports were used for oligonucleotide synthesis.

10

#### **Example 4: Construction of derivatized polystyrene support**

##### **Method A: Construction of support (13), Fig. 1.**

Several portions of a cross-linked polystyrene support (Mono R) (from Pharmacia) (0,50 g) were dried by coevaporation with pyridine and suspended in pyridine (2 ml). Different volumes of compound (3) (Scheme 1), prepared at a 1 mmol scale in pyridine (10 ml), see Example 1 above, were added to the above suspensions and the mixtures were shaken at 20°C for 20 hr. All activated sites that could theoretically form in the reaction of unreacted (2) with the solid support were quenched by the addition of methanol (5 ml). After 1 hr shaking, the mixture was filtered, dried by washing with dry pyridine, and transferred back to the stoppered flask, where 25 unreacted hydroxyl groups were capped with  $\text{Ac}_2\text{O}/\text{DMAP}$  (4-dimethylaminopyridine)/pyridine for 2 hrs. The amounts of DMTr thymidine bound directly to the support via the disiloxyl bond (13) were checked spectrophotometrically as above.

30

##### **Method B: Construction of support (14), Fig. 1**

A polystyrene support (10 mg), derivatized with hydroxyalkyl groups, was packed into cassettes for synthesis of oligonucleotides on a Gene Assembler Plus instrument 35 (Pharmacia). These packed supports were subjected to one coupling cycle using a standard thymidine amidite that was 5

times more diluted than recommended in standard coupling procedures. The support was extensively capped (10 min) on the machine operating in a manual mode. Under the above conditions the DMTr release experiments gave values comparable to those from the original 0.2  $\mu$ mol supports. All supports were further derivatized by coupling 9 consecutive thymidine nucleotides, followed by coupling of the appropriate amidite (5) from Example 2 above. These couplings were performed using standard amidite concentrations and synthesis protocols.

**Example 5: Solid-phase synthesis of oligodeoxynucleotides (15), Fig. 1**

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**15 Using the ABI 394 DNA Synthesizer and CPG supports**

The CPG support (14) described above was used for oligonucleotide synthesis. All couplings were performed using amidites protected by a benzoyl group at the exocyclic amine functions, under conditions recommended by the manufacturer for 0.2  $\mu$ mol scale synthesis, except that nucleoside amidites were used at half the recommended concentrations. The final DMTr groups were left on the synthesized oligonucleotide.

**25 Synthesis on a Gene Assembler (Pharmacia) using the polystyrene support**

All syntheses were done at the 0.2  $\mu$ mol scale using PAC amidites (Pharmacia Biotech AB), according to the manufacturer's instructions and without any changes in the recommended amidite concentrations. Supports constructed according to methods A (13) or B (14) in Example 4 above were used, see also Fig. 1.

Normal scale reaction of standard nucleoside amidites or the modified amidite (5) with hydroxyalkyl-polystyrene support resulted in a very high loading. Quantitative analysis of

the released DMTr-group revealed loadings of as much as 250 to 290  $\mu\text{mol/g}$ . Also functionalizations involving an excess of reagent (3) gave a relatively high degree of substitution. Reaction of 0.5 mmol of (3) per 1 g of solid support at 20°C for 2 hrs resulted in a support loaded at 180  $\mu\text{mol/g}$ . These comparatively large numbers were a consequence of the high density of hydroxyl groups on the support and of the high reactivity of the reagents used. Such highly derivatized supports are valuable for large-scale synthesis of short therapeutic oligonucleotides. For the synthesis of relatively long oligonucleotides measures had to be taken to limit this high degree of substitution. A satisfactory loading (38  $\mu\text{mol/g}$ ) could be obtained by using only 0.1 mmol of (3) per 1 g of support. Several-fold dilution of (5) under the recommended 0.1 M concentration was the best method for direct incorporation of (5) on the polystyrene support. The same dilution method was applied to the first standard amidite used for synthesis of the polystyrene version of the support (14). All coupling reactions described above were followed by an extensive capping procedure to block any unreacted hydroxyl groups.

**Example 6: Deprotection and purification of the synthetic oligodeoxynucleotides**

25 -----  
Different deprotection procedures were used, depending on the type of support that was applied in the synthesis.

**CPG-anchored oligonucleotides.** A syringe filled with a mixture of  $\text{Et}_3\text{N}/\text{EtOH}$  1:1 v/v was connected to a cassette containing support for oligonucleotide synthesis. Treatment of the support with base proceeded for 3 hrs at 20°C, with the occasional addition of a new aliquot of the solvent to the cassette. The support was washed with ethanol (2 ml), water (2x2 ml), dried with acetonitrile (3x2 ml), and after opening the cassette the solid support was transferred to a

Sarstedt screw-lock tube. Tetrabutyl ammonium fluoride (TBAF) 0.5 M in dry tetrahydrofuran (THF) (200  $\mu$ l) was added and the mixture was incubated for 4 hr at 20°C. The cleavage of the disiloxyl linker could alternatively be done using 5 200 ml of 0.5 M TBAF in dry DMF at 65°C for 30 min. Concentrated aq.  $\text{NH}_3$  (2 ml) was introduced and the mixture was placed in a 65°C oven for 12 hrs. After partial concentration the oligonucleotide was desalted on a NAP 10 Sephadex column (Pharmacia Biotech AB) and analyzed by HPLC 10 on a RP 18 column. Preparative runs were done on an FPLC using a reversed-phase Pep RPC column. Care was taken not to fractionate the hydrophobic trityl-containing product but rather to collect the whole peak, which closely resembles separations on disposable RP cartridges (Sep-Pak,  $\text{C}_{18}$ , 15 Waters, USA). After evaporation, the final removal of the DMTr groups was done using 80% aq. acetic acid for 20 min at 20°C, with subsequent evaporation of the acid. Alternatively, oligonucleotides phosphorylated at their 5'-position by the Tr-S phosphorylating reagent were finally deprotected 20 according to the published procedure [Connolly, (1987) Tetrahedron Lett., 28(4), 463-466].

**Polystyrene support-anchored oligonucleotides.** After completed synthesis, the support was transferred from the 25 cassette into a Sarstedt tube and subjected to treatment with concentrated aq.  $\text{NH}_3$  for 90 min at 65°C. After cooling, the particles were briefly centrifuged and the upper liquid phase was removed. The solid-support was washed 3 times with 2 ml of water and dried by washing with acetonitrile. Two 30 hundred  $\mu$ l of 0.5 M TBAF in THF was added and the mixture was incubated for 4 hr. Finally, the mixture was diluted with 0.8 ml water and the oligonucleotide was desalted on a NAP 10 Sephadex column. The further deprotection and purification steps follow exactly those described for CP3- 35 bound oligonucleotides.

**Example 7: Electrophoretic analysis of oligonucleotides**  
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Oligonucleotides to be analyzed electrophoretically were  
5 labelled with  $^{32}\text{P}$  at their 5'-end using polynucleotide  
kinase in a 50  $\mu\text{l}$  reaction volume of 50 mM KAc, 10 mM  $\text{MgCl}_2$ ,  
10 mM Tris-HAc (pH 7.5), 10  $\mu\text{Ci}$  ( $\gamma$ - $^{32}\text{P}$ )ATP (3000 Ci/mmol),  
and 10 U polynucleotide kinase (Amersham) at 37°C for 30 min.  
The labelling reaction was stopped by desalting on a  
10 Sephadex G-50 spin column, followed by incubation at 65°C for  
5 min. All oligonucleotides analyzed electrophoretically in  
this study were synthesized with a 5'-phosphate to ensure  
that cleaved apurinic oligonucleotides would label with the  
same efficiency as the full-length molecules. After  
15 separation on a denaturing 6% polyacrylamide gel the  
radioactivity was recorded by autoradiography (Amersham  
Hyperfilm) or, for quantitative measurements of band  
intensities, scanned on a Phosphorimager instrument  
(Molecular Dynamics).

20

**Example 8: Purine-rich oligonucleotides**  
-----

The risk of depurination of an oligonucleotide increases  
with the number of purines and the total length of the  
25 oligonucleotide. An increased amount of purines in an  
oligodeoxynucleotide gives a high probability of its  
depurination and the following break-down. To show the  
capability of the new method to eliminate these short  
sequences, two 81-mer (AG) $_{40}$ T sequences were synthesized in  
30 parallel using standard and novel CPG-based supports (14),  
respectively. Partially deprotected 5'-DMTr-substituted  
oligonucleotides were analyzed and isolated by HPLC. Both  
products were detritylated, 5'- $^{32}\text{P}$  kinased and  
electrophoretically separated on a denaturing polyacrylamide  
35 gel. The results, presented in Fig. 2 show the superiority  
of the invention compared to prior art. This superiority

could already be anticipated by comparing the shapes of the HPLC chromatogram (A). The much broader peak obtained in the standard synthesis reflects the presence of the shorter and therefore more hydrophobe tritylated fragments. It is clear from the scanned presentation of the gel separation (B) that the product synthesized according to the present invention is practically free of all truncated and depurinated sequences. Moreover, this material contains substantially lesser amount of n-1 fragments [(D) compared to (C)].

10

#### Example 9: Circularization of a padlock probe

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A 91-mer oligonucleotide (M13C91: 5'-P-  
15 GCCTGCAGGTCGACTCTAGA(T)<sub>50</sub>CGGCCAGTGCCAAGCTTGCA-3') were synthesized according to the invention in order to test how it would work as a padlock probe [Nilsson et al (1994), supra] that is able to circularize in presence of an oligonucleotide template (M1350comp: 5'-  
20 TTTTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTTTTT-3') and a DNA-ligase. The ligation reaction was performed using 0.3 pmol of 5'-labelled probe and 5.5 pmol of template in a volume of 10 µl 20 mM Tris-HCl ( pH 8.3), 25 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM NAD, 0.01% Triton X-100 and 10 U of Tth DNA  
25 ligase. The sample were subjected to one, two or three cycles of 94°C for 15 seconds and 55°C for 10 min. The reactions were cooled on ice and stopped by adding 10 µl of loading buffer containing 50 % formamide and 10 mM EDTA followed by a incubation at 65°C for 10 min. The reactions  
30 were analyzed on a denaturing polyacrylamide gel as above.

The result of the ligation is shown in Fig. 3 which clearly shows that all full-length oligonucleotides are ligatable as well as most of the n-1 products, as would be expected if  
35 the deletions were evenly distributed throughout the sequence. It also shows the presence of many shorter

products that are ligatable and thus having intact 5'- and 3'- ends thereby revealing deletion events of more than one nucleotide. Consequently, all nonligating shorter sequences must contain deletions that prevent effective hybridization to the targeted DNA.

The present invention delivers products free of truncated and post-depurination cleaved fragments, moreover, the isolated products contain much less n-1 fragments. The amount of this n-1 material was estimated, in a study performed on a short 5-mer sequence and using a standard synthesis protocol ([Iyer et al, (1995), 14(6) 1349-1357]), to be roughly 3 to 5 %. By applying pre-cap procedure according to Iyer et al and short contact time of ammonia with the CPG support it was possible to lower this figure to 1.5 to 2 %. In contrast, in an experiment according to the present invention for synthesis of a 20-mer using CPG support (14), the amount of contaminating n-1 sequences was found to be 1.9 %.

The support system of the present invention is not limited to the solid-phase synthesis of oligonucleotides described above. Another contemplated possibility is to use it as a linker between support and hydroxyl containing components in the combinatorial chemistry ([Plunkett et al, (1995), J. Org. Chem., 60(19), 6006-6007]).

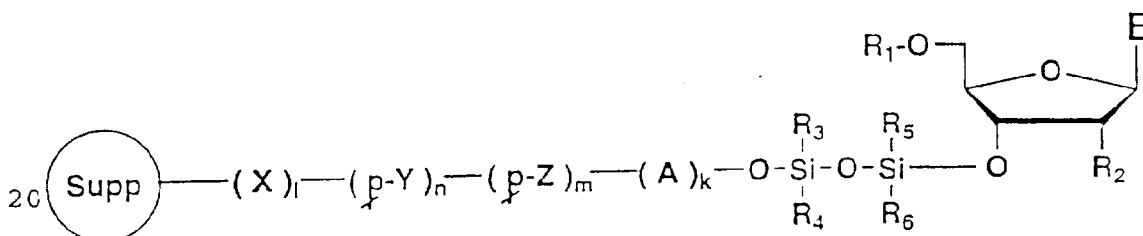
## CLAIMS

1. A support system, for solid phase synthesis of oligomers, comprising a support, a linker and a starting  
5 compound of the oligomer, characterized in that the starting compound of the oligomer is bound to the support via a disiloxyl linkage.

2. A support system according to claim 1, characterized  
10 in that the starting compound is a nucleoside and the synthesis is an oligonucleotide synthesis.

3. A support system according to claims 1 or 2, characterized by having the formula

15



wherein

B is a nucleoside or deoxynucleoside base; R<sub>2</sub> is -H, -OH, or  
25 OR<sub>7</sub> in which R<sub>7</sub> is a protecting group; R<sub>1</sub> is a protecting group; R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub>, R<sub>6</sub> taken separately each represent alkyl, aryl, cycloalkyl, alkenyl, aralkyl, cycloalkylalkyl, alkyloxy, aryloxy, cycloalkyloxy, alkenyloxy and aralkyloxy; Supp is a solid support; X is an anchoring group used for  
30 covalent bonding to the support;

(p-Y)<sub>n</sub> and (p-Z)<sub>m</sub> are oligophosphotriester linkers, wherein p represents a phosphotriester, Y and Z are independently selected from a nucleoside and a rest of a diol, A is an aliphatic or aromatic group, n is a number between 0-50,  
35 preferably 0-10, and k, l, m are each a numbers of 0 or 1, with the proviso that when m and n are 0 then l and k are 0



and with the proviso that when  $m > 0$  then  $k$  is 0 and  $X$  is O or S.

4. A support system according to claim 3, characterized in that the anchoring group is O, S or an amide function.

5

5. A support system according to any one of the above claims, characterized in that  $R_1$  is a trityl, monomethoxy trityl, dimethoxytrityl, pixyl or other higher alkoxy-substituted trityl-protecting groups, and  $E$  is adenine, 10 guanine, cytosine, uracil, thymine, or inosine.

6. A support system according to any one of the above claims, characterized in that  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$  are isopropyl.

15 7. A method for synthesis of oligonucleotides on a solid support, characterized by the following steps:

(i) preparing a support system as defined in claim 3;  
(ii) condensation of nucleotides onto the first nucleoside of the support system to synthesize an oligonucleotide;

20 (iii) removal of all protecting groups on the oligonucleotide except the 5'-protecting group, and cleavage of apurinic sites formed during acid-catalysed deprotection;  
(iv) cleavage of the full length product from the support; and

25 (v) purification of the oligonucleotide.

8. A method according to claim 7, characterized in that in step (i) an oligophosphotriester linker  $(p-Y)_n$  is synthesized on the solid support and that a starting 30 nucleoside is bound to the linker via a  $(p-z)_m$  linker and a disiloxyl group.

9. A method according to claim 7, characterized in the disiloxyl linkage is cleaved selectively with tetra alkyl 35 ammonium fluoride before step (v) and the purification is performed by reversed phase chromatography, using the 5'-protecting group as an affinity handle.

10. A method according to claim 7, characterized in step (v) is performed by exonuclease treatment whereby non-protected oligonucleotides will be digested.

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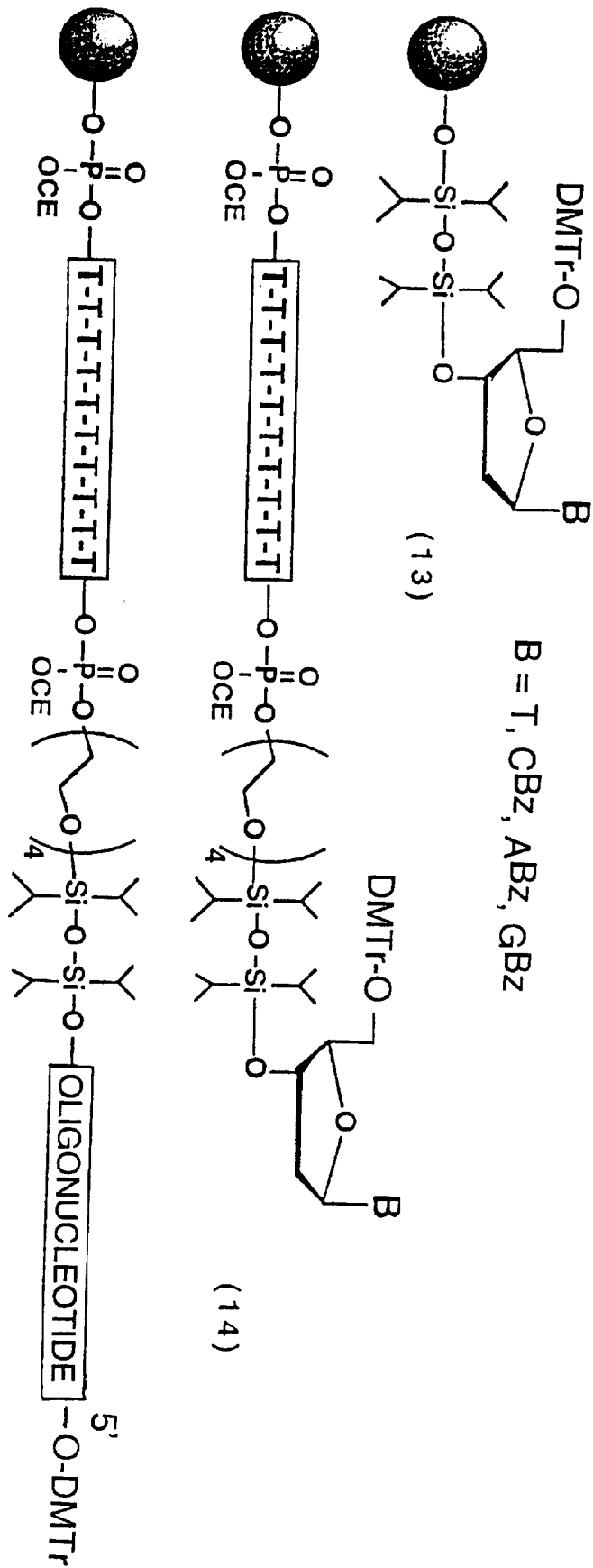


FIG. 1

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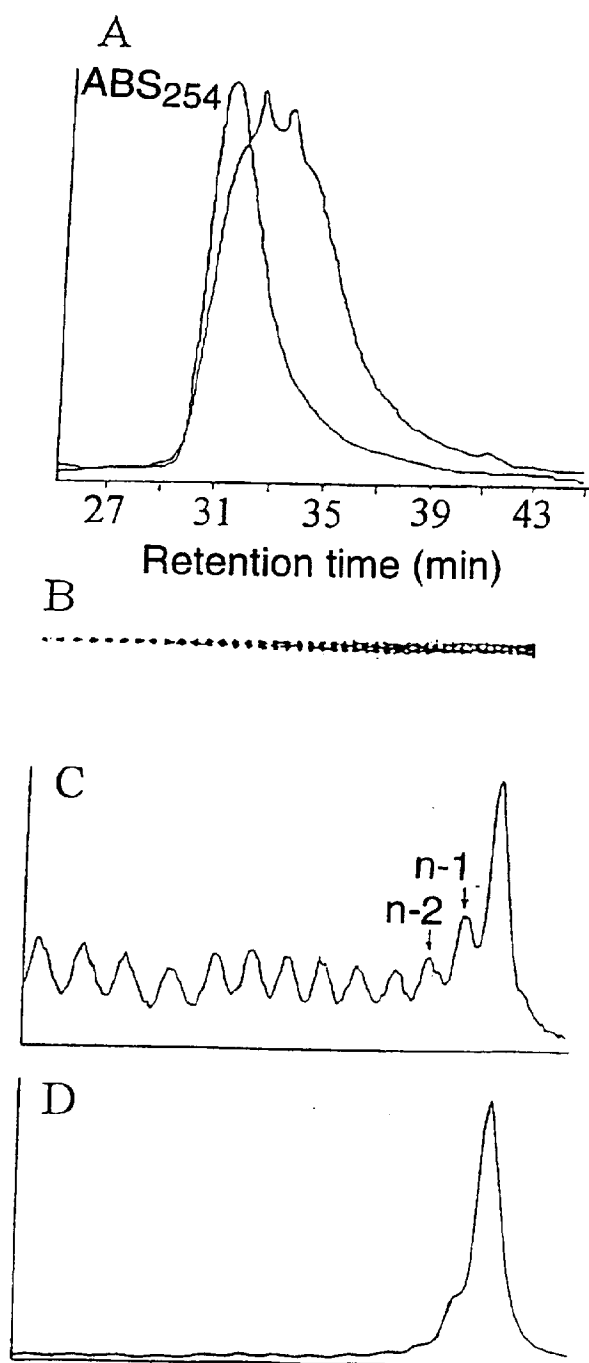


FIG. 2

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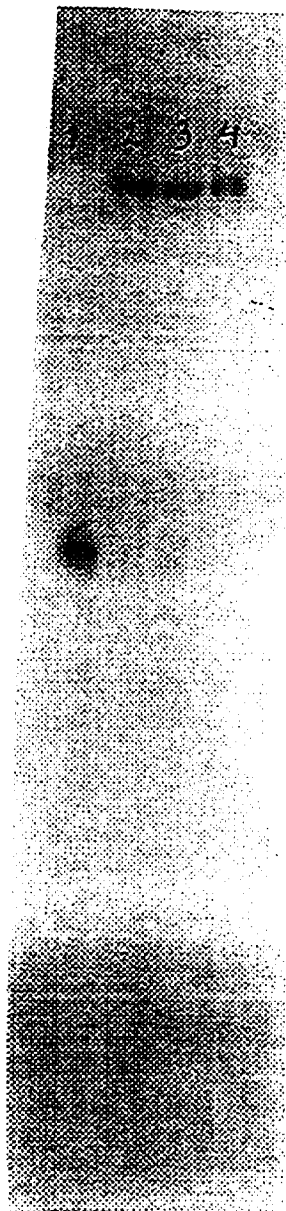


FIG. 3

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 97/01418

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07H 21/00, C07H 21/02, C07H 21/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9209615 A1 (PHARMACIA LKB BIOTECHNOLOGY AB), 11 June 1992 (11.06.92) --	1-10
A	WO 9313220 A1 (TEPNEL MEDICAL LIMITED), 8 July 1993 (08.07.93) --	1-10
A	WO 9204364 A1 (STERLING DRUG INC.), 19 March 1992 (19.03.92) -- -----	1-10

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&amp;" document member of the same patent family

Date of the actual completion of the international search

3 December 1997

Date of mailing of the international search report

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**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

01/10/97

International application No.  
PCT/SE 97/01418

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9209615 A1	11/06/92	AT 133684 T	15/02/96
		DE 69116864 D,T	12/09/96
		EP 0514513 A,B	25/11/92
		SE 0514513 T3	
		ES 2084839 T	16/05/96
		JP 5503715 T	17/06/93
		SE 9003743 D	00/00/00
		US 5589586 A	31/12/96
WO 9313220 A1	08/07/93	AU 679903 B	17/07/97
		AU 3169493 A	28/07/93
		BR 9206985 A	05/12/95
		CA 2126748 A	25/06/93
		EP 0620863 A	26/10/94
		FI 943054 A	22/08/94
		JP 7502405 T	16/03/95
		NO 942399 A	12/08/94
		NZ 246203 A	25/09/96
		PL 171810 B	30/06/97
		ZA 9210008 A	03/08/93
WO 9204364 A1	19/03/92	AT 116328 T	15/01/95
		AU 651289 B	14/07/94
		AU 8533991 A	30/03/92
		CA 2091559 A	13/03/92
		DE 69106376 D,T	13/07/95
		EP 0548189 A,B	30/06/93
		SE 0548189 T3	
		ES 2067246 T	16/03/95
		HU 64555 A	28/01/94
		IL 99451 A	31/12/95
		JP 6506910 T	04/08/94
		US 5214134 A	25/05/93